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Small intestine neuromuscular dysfunction in a mouse model of dextran sulfate sodium-induced ileitis: Involvement of dopaminergic neurotransmission

Silvia Cerantola [a,1], Sofia Faggin [a,1], Valentina Caputi [a,b], Annalisa Bosi [c], Davide Banfi [c], Anna Rambaldo [d], Andrea Porzionato [d], Rosa Di Liddo [a], Raffaele De Caro [d], Edoardo V. Savarino [e], Cristina Giaroni [c], Maria Cecilia Giron [a,f,*]

[a] Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Padova, Italy
[b] Department of Poultry Science, University of Arkansas, Fayetteville, AR 72704, USA
[c] Department of Medicine and Surgery, University of Insubria, Varese, Italy
[d] Department of Neuroscience, University of Padova, Padova, Italy
[e] Department of Surgery, Oncology and Gastroenterology, University of Padova, Padova, Italy
[f] IRCCS San Camillo Hospital, 30126 Venice, Italy

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ABSTRACT

Aims: Anomalies in dopaminergic machinery have been shown in inflammatory bowel disease (IBD) patients and preclinical models of IBD. Thus, we aimed to evaluate the impact of dextran sodium sulfate (DSS)-induced ileitis on enteric dopaminergic pathways.

Materials and methods: Male C57/Bl6 mice (10 ± 2 weeks old) received 2% DSS in drinking water for 5 days and were then switched to regular drinking water for 3 days. To measure ileitis severity inflammatory cytokines (IL-1β, TNFα, IL-6) levels were assessed. Changes in ileal muscle tension were isometrically recorded following: 1) cumulative addition of dopamine on basal tone (0.1–1000 μM); ii) 4-Hz electric field stimulation (EFS) in the presence of 30 μM dopamine with/without 10 μM SCH-23390 (dopamine D1 receptor (D1R) antagonist) or 10 μM sulpiride (D2R antagonist). Immunofluorescence distribution of the neuronal HuC/D protein, glial S100β marker, D1R, and dopamine transporter (DAT) were determined in longitudinal-muscle-myenteric plexus whole-mounts (LMMPs) by confocal microscopy. D1R and D2R mRNA transcripts were evaluated by qRT-PCR.

Key findings: DSS caused an inflammatory process in the small intestine associated to dysmotility and altered barrier permeability, as suggested by decreased fecal output and enhanced stool water content. DSS treatment caused a significant increase of DAT and D1R myenteric immunoreactivity as well as of D1R and D2R mRNA levels, accompanied by a significant reduction of dopamine-mediated relaxation, involving primarily D1-like receptors.

Significance: Mouse ileitis affects enteric dopaminergic neurotransmission mainly involving D1R-mediated responses. These findings provide novel information on the participation of dopaminergic pathways in IBD-mediated neuromuscular dysfunction.

Abbreviations: DRs, dopaminergic receptors; CRC, colorectal cancer; ENS, enteric nervous system; IBD, inflammatory bowel disease; LMMPs, longitudinal-muscle-myenteric plexus whole mounts; DAT, dopamine transporter; DSS, dextran sodium sulfate; EFS, electric field stimulation; H&E, hematoxylin/eosin; DAI, disease activity index; CD, Crohn's disease; UC, ulcerative colitis; PD, Parkinson's disease.

* Corresponding author at: Pharmacology Building, Department of Pharmaceutical and Pharmacological Sciences, Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Largo Meneghetti, 2, 35131 Padova, Italy.
E-mail address: cecilia.giron@unipd.it (M.C. Giron).
[1] Authors contributed equally to this study.

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1. Introduction

Dopamine, one of the main endogenous catecholamines, is considered an intrinsic neurotransmitter in the enteric nervous system (ENS), playing an important role in maintaining gut homeostasis [1,2]. Several subtypes of dopaminergic receptors (DRs) have been localized in several cells constituting the enteric microenvironment, including neuronal and non-neuronal cells (e.g., epithelial, immune and muscle cells) along the gastrointestinal tract [2–7]. DRs belong to the G protein–coupled receptor family and are grouped in two main families: D1-like receptors (i.e., D1R and D5R) and D2-like receptors (i.e., D2R, D3R, and D4R) [8,9]. Dopamine may have different effects on intestinal motor function, concurring to modulate both excitatory and inhibitory responses, producing contraction of the circular muscle layer, via D1-like receptors, and relaxation of the longitudinal muscle layer, via D2-like receptors [10]. Such dual role may depend upon the different localization of dopamine receptors in the gut, since D1Rs are mainly postjunctionally located, whereas D2Rs are located both at pre/postjunctional sites, and changes in their expression may affect gastrointestinal motility [11]. Data obtained from both clinical and preclinical studies, however, prevalently address dopamine as an inhibitory enteric neurotransmitter modulating gastrointestinal relaxation [2–4,7,12,13]. Furthermore, the dopaminergic system controls gastrointestinal transit by cooperating with other neurotransmitter pathways, such as serotoninergic, adenosinergic and GABAergic pathways [14,15].

Currently, several dopaminergic antagonists, such as domperidone, haloperidol, and sulpiride, are used for the management of several gastrointestinal motor disorders [2,10,11]. The inhibition of D2R with antidopaminergics (e.g., domperidone, metoclopramide) provokes a gastroprokinetic response. This effect is mediated by the prejunctional D2R expressed on intrinsic cholinergic nerves which negatively modulate acetylcholine release [16].

Dopamine agonists appear to play an important protective role during colitis regardless of the experimental model [17]. Besides acting as a neurotransmitter, dopamine concurs in regulating immunity by interacting with its receptors expressed on innate and adaptive immune cells, suggesting an involvement in autoimmune disorders and, even, cancer [18–20]. In addition to control gut contractility, this catecholamine reduces VEGF-mediated vascular permeability and has an angiogenic action in models of colorectal cancer (CRC) with concomitant inhibition of tumor growth. Human polymorphisms in D2R have been identified and associated to a higher risk of CRC whereas a marked reduction of dopamine content has been linked to CRC progression [14,18–22]. In particular, D3R activation dampens the immunosuppressive potency of Tregs, their gut tropism and IL-10 production whereas D5R stimulation leads to higher expression of ROR-γt in dendritic cells as well as release of IL-23 and IL-12. Moreover, changes in dopaminergic pathways have been linked to promote CRC, which patients affected by inflammatory bowel disease (IBD) are at a higher risk of developing [22].

In IBD patients, significant reductions in dopamine tissue levels were detected, highlighting that the inflammatory process may impair enteric dopamine synthesis and storage. Decreased dopamine content has been related to the manifestation of IBD-related diarrhea, suggesting that the catecholamine has an active role in intestinal secretion electrolyte balance [17,23,24]. Interestingly, IBD and Parkinson's disease (PD) appear to share a common pathophysiological and genetic link [25,26].

In this study, to investigate the potential role of the enteric dopaminergic neurotransmission in intestinal inflammation, we evaluated morphological and functional changes in the mouse ENS, after an acute ileitis caused by a 5-day treatment with dextran sodium sulfate (DSS). DSS-induced inflammation is considered a valuable and reproducible model for understanding the molecular mechanisms underlying IBD since animals developed signs of inflammation along all the gastrointestinal tract with many features similar to IBD in humans [27–29].

2. Materials and methods

2.1. Chemicals

Unless otherwise specified, all chemicals were obtained from Sigma–Aldrich (Milan, Italy) and were of the highest commercially available analytical grade. PFA and Citifluor-1 were acquired from Electron Microscopy Sciences-Società Italiana Chimici (Rome, Italy), whereas Triton-X-100 was purchased from Applichem (Milan, Italy). All drugs for in vitro contractility studies were dissolved in milliQ water.

2.2. Animals and in vivo treatment

All animal care and experimental protocols were approved by the Animal Care and Use Ethics Committee of the University of Padova and by the Italian Ministry of Health (authorization number: 1142/2015-PR) and were performed in accordance with national and EU guidelines for the handling and use of experimental animals. Animal studies are reported in compliance with the ARRIVE guidelines [30].

Male wild-type mice (WT; C57BL/6J; 10 ± 2 weeks old; Charles River Laboratories, Italy) were housed in individually ventilated cages (IVC) at the Animal Facility of the Department of Pharmaceutical and Pharmacological Sciences, University of Padova. All animals were maintained under controlled environmental conditions (temperature 21 ± 1 °C; relative humidity 60–70%) with a regular 12/12 h light/dark cycle, free access to standard laboratory chow and tap water ad libitum. To induce experimental ileitis WT mice were treated for 5 days with dextran sodium sulfate (DSS; 2% wt/vol; MW: 40,000 Da) added to the drinking water and then switched to regular drinking water for the next 3 days of wash-out (DSS-treated group) [31]. Control (SHAM) mice were similarly treated with vehicle (tap water). The status of mice was monitored every day by measuring body weight, general status and the development of diarrhea and/or hematochezia. At the end of treatment on day 8, animals were sacrificed by cervical dislocation to preserve gut contractility [32]. All the subsequent experimental procedures were conducted blindly. The disease activity index (DAI) was determined by attributing well-established and validated scores for signs that are similar to the clinical presentation of human IBD (i.e., body weight loss, stool consistency and rectal bleeding) [33].

2.3. Histopathology

Ileal (2–6 cm from ileocecal valve) and colonic (1–5 cm from the caecum) specimens were sampled and cut in multiple transverse sections. Samples were fixed in 10% buffered formalin and paraffin-embedded. Five-μm-thick sections were stained with hematoxylin/eosin (H&E) and blindly assessed for inflammation and necrosis; for each animal, 6–8 sections were evaluated [34,35].

Ten-μm-thick sections of the ileum were also immunostained by anti-dopamine transporter antibody (GTX133152, rabbit polyclonal antibody, Genetex; dilution of 1:4000; antigen retrieval with low pH Envision TM FLEX, low pH, K8005 buffer) [36,37].

2.4. Pellet frequency and fecal water content

Fecal pellet output was daily examined in both SHAM and DSS mice. Animals were individually placed in a novel environment (clean cage) and observed for 60 min. The number of pellets per mouse was collected and weighed (wet weight). Pellets were subsequently dried overnight at 65 °C and weighed again to obtain the dry weight. Fecal water content was measured as the difference between wet and dry weights [38].

2.5. RNA isolation and quantitative RT-PCR

To evaluate the influence of DSS-induced ileitis on IL-6, IL-1β, TNFα, D1R, and D2R mRNA levels, total RNA from mice mucosa-deprived

small intestine wall was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (DNase Free, Ambion) to remove possible traces of contaminating DNA. 2 μg of total RNA was retrotranscribed using the High-Capacity cDNA synthesis kit (Applied Biosystems, Life Technologies, Grand Island, NY, USA). Quantitative RT-PCR was performed on the QuantStudio 3 Real-Time PCR Systems (Thermo Fisher Scientific, Carlsbad, California) with Power Sybr Green Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions. Primers were designed to have a similar amplicon size and similar amplification efficiency as required for the utilization of the $2^{-\Delta\Delta Ct}$ method to compare gene expression [4,39,40], using Primer Express software (Applied Biosystems, Foster City, CA, USA) on the basis of available sequences deposited in public database. Primers are reported in Table 1. For quantitative RT-PCR, a final concentration of 500 nm for each primer was used and experiments were performed at least 7 times for each experimental group, as previously described [41]. $2^{-\Delta\Delta Ct}$ values obtained from the comparison between normalized Ct values of DSS-treated samples with those obtained from control samples were used to evaluate the effect of DSS-induced ileitis on IL-6, IL-1β, TNFα, D1R, and D2R mRNA levels in the small intestine [42].

2.6. In vitro contractility studies

Contractility experiments were conducted as previously described [4,42,43]. Briefly, longitudinal segments (1 cm) from distal ileum were mounted along the longitudinal axis in 10-mL-organ baths and allowed to equilibrate for 45 min in Krebs solution (NaCl 118 mM, KCl 4.7 mM, $CaCl_2{\cdot}2H_2O$ 2.5 mM, $MgSO_4 \cdot 7xH_2O$ 1.2 mM, K_2HPO_4 1.2 mM, $NaHCO_3$ 25 mM, $C_6H_{12}O_6$ 11 mM) maintained at 37 °C with 95% O_2/5% CO_2. Changes in muscle tension were recorded by isometric transducers (World Precision Instruments, Berlin, Germany) connected to a PowerLab 4/30 system (ADInstruments, Oxford, United Kingdom).

To determine changes in dopaminergic response concentration-dependent curves were made by non-cumulative addition of dopamine (0.1–1000 μM) on basal tone [4]. In a separate set of experiments, the amplitude of ileal spontaneous contractions was evaluated in the absence or presence of 10 μM SCH-23390 (D1R antagonist) or 10 μM sulpiride (D2R antagonist). In order to evaluate the role of D1R and D2R on the neuromuscular response, 4 Hz-EFS stimulation was performed before and after the addition of 30 μM dopamine in absence or presence of 10 μM SCH-23390 or 10 μM sulpiride. The antagonist concentrations used were based on the pKi described in the literature, and the dopaminergic antagonists were allowed to maintain contact with the tissue for at least 20 min [2,4,12]. Contractile responses were expressed as gram tension/g dry tissue weight of isolated ileal segments and ileal relaxation was calculated as AUC and normalized per g dry tissue weight [42].

2.7. Immunohistochemistry on mouse ileal whole mount preparations

Immunohistochemistry studies were performed as previously described [4]. To evaluate changes on ENS structure, fresh isolated distal ileum segments (10 cm) were rinsed with PBS and exposed to fixative solution, composed by 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), for 2 h at room temperature. Tissues were separated into two layers under a dissecting microscope: the outer musculature with adhering serosa and the submucosa/mucosa. The circular muscle was removed to obtain longitudinal muscle-myenteric plexus whole-mount preparations (LMMPs). LMMPs from SHAM and DSS mice were pinned down on the bottom of Sylgard-coated dishes and washed in PBT (PBS with 0.3% Triton X-100) for 45 min with gentle shaking. After blocking nonspecific sites with 5% bovine serum albumin (BSA) in PBT for 1.5 h at room temperature, LMMPs were incubated overnight at room temperature with the following primary antibodies diluted in PBT and BSA 5%: mouse biotin-conjugate HuC/D (1:100, Thermo Fisher Scientific, cat#: A-21272), guinea pig anti-S100β (1:100, Synaptic Systems, cat#:28700), rabbit anti-D1R (1:50, Abcam, cat#: ab20066), rabbit anti-DAT (1:50, GeneTex, cat#: GTX133152). The following day, LMMPs preparations were washed in PBS and incubated for 2 h at room temperature with the following secondary antibodies, diluted in PBT and BSA 5%: streptavidin Alexa 555-conjugated (1:1000, Thermo Fisher Scientific, cat#: S21381), anti-rabbit Alexa 488 (1:1000, Thermo Fisher Scientific, cat#: A-11008), anti-guinea pig Alexa 488 (1:1000, Thermo Fisher Scientific, cat#: AB_2534117). LMMPs preparations were mounted on glass slides using a Mowiol mounting medium. Negative controls were obtained by incubating sections with isotype-matched control antibodies at the same concentration as primary antibody and/or pre-incubating each antibody with the corresponding control peptide (final concentration as indicated by manufacturer's instructions).

Table 1
Sequence of primers used for the qRT-PCR analysis.

Gene	Sequence
β-Actin	F 5′-ACCAGAGGCATACAGGGACA-3′ R 5′-CTAAGGCCAACCGTGAAAAG-3′
IL-6	F 5′-TAGTCCTTCCTACCCCAATTTCC-3′ R 5′-TTGGTCCTTAGCCACTCCTTC-3′
IL-1β	F 5′-GCAACTGTTCCTGAACTCAACT-3′ R 5′-ATCTTTTGGGGTCCGTCAACT-3′
TNFα	F 5′-CCCTCACACTCAGATCATCTTCT-3′ R 5′-GCTACGACGTGGGCTACAG-3′
D1R	F 5′-GTAGCCATTATGATCGTCAC-3′ R 5′-GATCACAGACAGTGTCTTCAG-3′
D2R	F 5′-GCAGCCGAGCTTTCAGGGCC-3′ R 5′-GGGATGTTGCAGTCACAGTG-3′

2.8. Confocal image acquisition and analysis

Images were acquired with the Zeiss LSM 800 confocal imaging system (Oberkochen, Germany) equipped with an oil-immersion 63× objectives (NA 1.4) used for the LMMP preparation. Z-series images (25 planes) of 512 × 512 pixels were processed as maximum intensity projections for LMMP whole mount preparations. All microscope settings were maintained constant for all images. In ileal LMMPs, the analysis of the total neuron population was performed by counting HuC/D^+ cells in 10 randomly chosen images per mouse. The total number of HuC/D^+ neurons was measured in each image and normalized per myenteric ganglion area, as previously described [34,38,44]. Changes in the immunoreactivity of the D1R, the dopamine transporter (DAT), and the glial marker S100β, were determined for each antigen by capturing 20 images per mouse, as previously described [34,38]. The intensity of staining for each antibody was expressed as the fluorescence intensity (density index) of labelling normalized per myenteric ganglion area and was reported as mean ± SEM [44].

2.9. Data and statistical analysis

The data and statistical analyses have been performed in compliance with the recommendations on experimental design and analysis in pharmacology [45]. All data are expressed as mean ± SEM and were analyzed by investigators blinded to the treatments, using GraphPad Prism software version 8.4 (San Diego, CA, United States). Animals were randomly divided into the two experimental groups. The distribution of data was tested with the Shapiro–Wilk normality test. Statistical significance was calculated with paired or unpaired Student's *t*-test for two-sample comparisons, one-way ANOVA followed by Newman–Keuls post hoc test for multiple comparison. The differences between groups were considered significant when $p < 0.05$; "*N*" values indicated the number of animals. Post hoc tests were run only if F achieved $p < 0.05$, and there was no significant variance inhomogeneity. Correlations between small intestine length and the DAI score were determined by Pearson correlation analysis.

3. Results

3.1. Assessment of intestinal inflammation and evaluation of ileal propulsive activity

The body weight of SHAM animals increased steadily after eight days (+4 ± 1%; $P > 0.05$; Fig. 1A). In contrast, after 8 day-treatment, DSS mice exhibited a significant weight loss (−10 ± 2%; $P < 0.01$; Fig. 1A) and enhanced DAI score (Fig. 1B). The presence of ileitis was characterized by a significant reduction of fecal pellet output per hour and by a marked increase water content in stools, both representing signs suggestive of a prolonged time of gastrointestinal transit associated with alterations on gut permeability [29,46](Fig. 1C and D). In DSS mice, the length of the small intestine was significantly reduced (−10 ± 1%, $N = 30$/group; $P < 0.05$; data not shown) as compared to SHAM mice, and negatively correlated with the DAI score (Pearson coefficient R = −0.84; $P < 0.001$; Cl95%: −0,9340 to −0,6441), highlighting the development of an inflammatory response [47]. Histopathological analyses of DSS-treated mice showed extensive areas of severe inflammation and necrosis of the colonic mucosa. In the ileal samples, areas of inflammation and necrosis were also identified, although less severe and less numerous. These lesions were characterized by crypt/villus damage, mucosal destruction, epithelial erosions, and infiltration of inflammatory cells (Fig. 2A). To further assess the presence of ileal inflammation, we analyzed mRNA levels of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 levels. DSS administration determined a 1.5-fold, 3-fold, and 2-fold increase of IL-1β, IL-6, and TNF-α mRNA levels, respectively, compared to SHAM mice (Fig. 2).

3.2. DSS-induced ileitis affects dopaminergic receptor-mediated response

Considering that dopaminergic pathways are affected by gut inflammation, we sought to determine the response of the mouse ileum longitudinal muscle to increasing dopamine concentrations (0.1–1000 μM) following DSS treatment. Addition of dopamine in our preparations evoked a concentration-dependent relaxation response, determining a decrement of spontaneous contractile amplitude until complete disappearance. The inhibitory effect of dopamine was significantly reduced in ileal segments from DSS-treated mice as compared to SHAM animals (ΔEmax = −33 ± 5%, $N = 6$/group; $P < 0.001$; Fig. 3A). The inhibitory effects persisted throughout the dopamine contact time and were reversible after 10-min wash-out. In Fig. 3B, original tracings are reported to display the concentration-dependent inhibitory effects of dopamine in ileal segments obtained from DSS and SHAM.

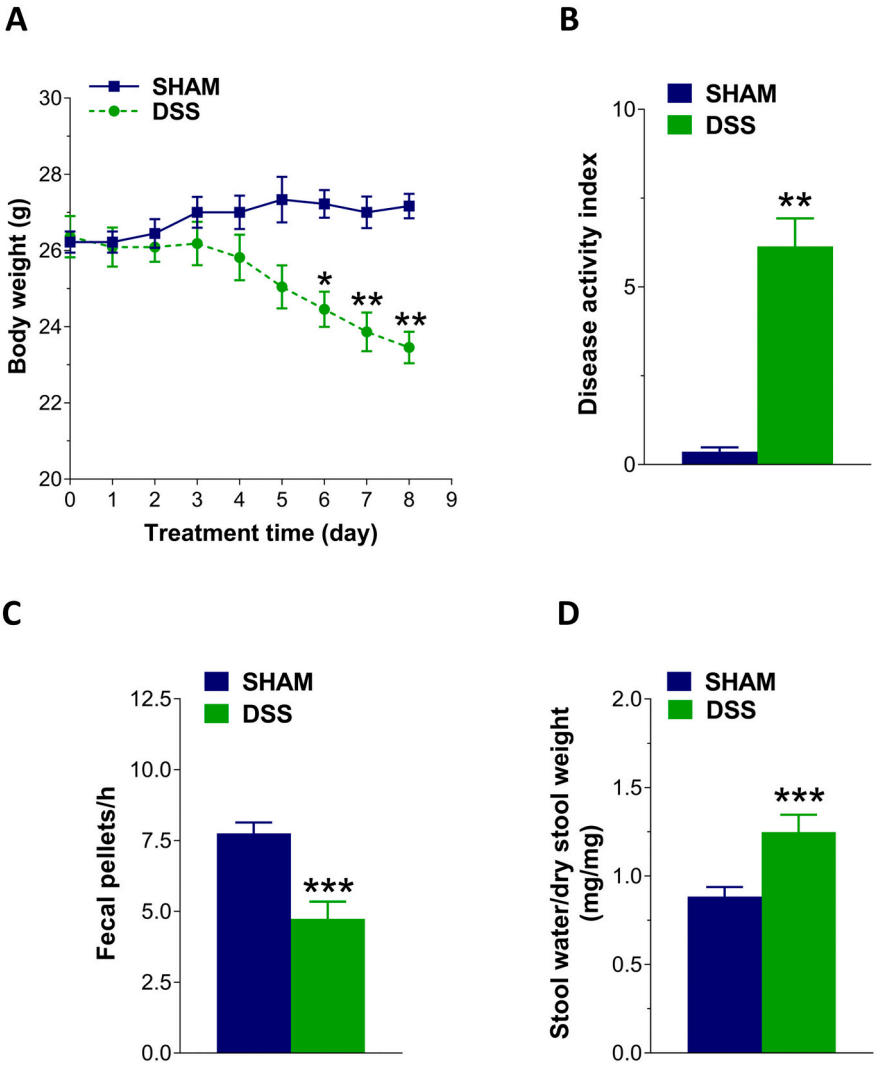

Fig. 1. DSS treatment determines weight loss and motor disturbances. A) Changes of body weight during 8 day-treatment in SHAM and DSS mice. B) Clinical assessment of DSS-induced ileitis. DAI was scored from each mouse for weight loss, stool consistency, and bleeding. C) Fecal pellet frequency per hour in SHAM and DSS mice. D) Stool water content in SHAM and DSS mice. Data are reported as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs SHAM mice. $N = 30$ mice/group.

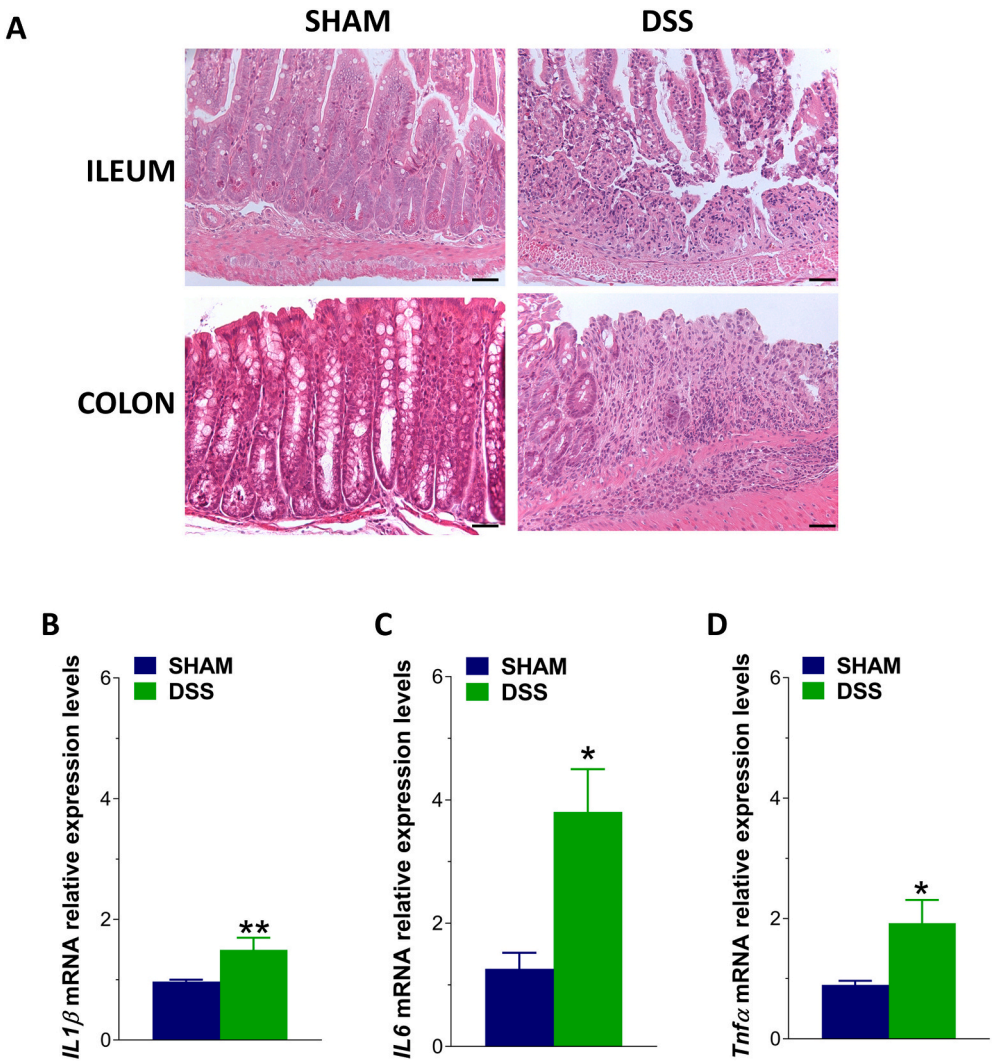

Fig. 2. DSS treatment causes inflammation in the small intestine. A) H&E-stained ileal sections from SHAM and dextran sulfate sodium (DSS)- treated mice. Scale bars = 50 μm. B–D) qRT-PCR quantification of *IL1β* (B), *IL6* (C), and *Tnfα* (D) mRNA levels in the small intestine of SHAM and DSS mice. Data are reported as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs SHAM mice. $N = 6$ mice/group.

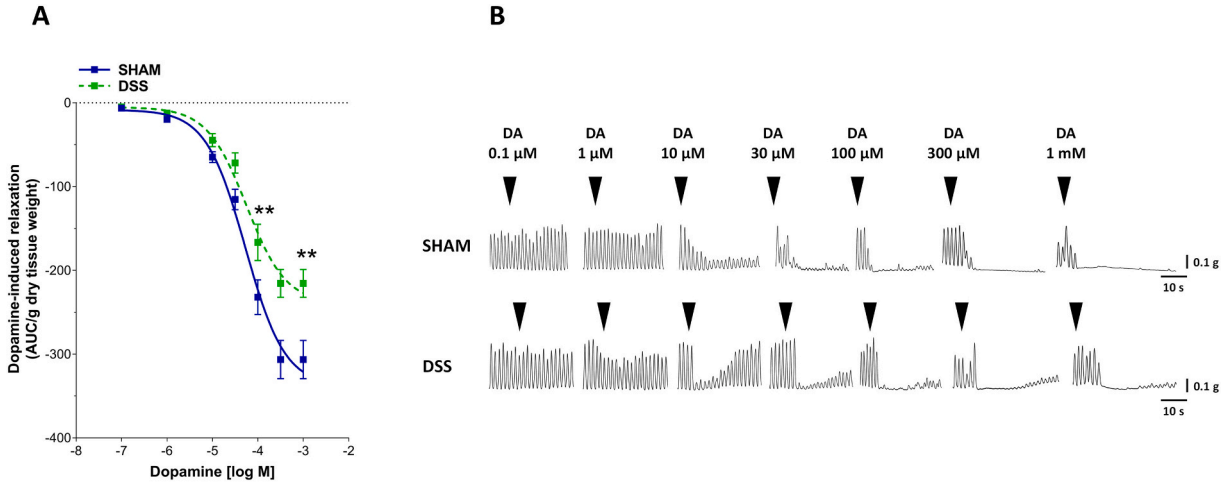

Fig. 3. DSS-induced ileitis affects dopaminergic neuromuscular response. A) Inhibitory concentration–response curves to dopamine (DA) on spontaneous contractile activity of isolated ileal preparations from SHAM and DSS mice. B) Representative tracings of responses induced by increasing DA concentration in SHAM and DSS preparations. Data are reported as mean ± SEM. $^{**}P < 0.01$ vs SHAM mice. $N = 6$ mice/group.

3.3. Influence of DSS-induced ileitis on D1 and D2 receptor response

In order to evaluate the possible involvement of D1R and D2R in dopamine-induced inhibition, we assessed the influence of the D1R antagonist, SCH-23390 (10 μM), and of the D2R antagonist, sulpiride (10 μM), on the dopamine on both SHAM and DSS mouse ileum longitudinal muscle (Fig. 4). Interestingly, SCH-23390, per se, significantly augmented the amplitude of spontaneous contractions to the same extent in SHAM and DSS preparations (Fig. 4A and B). Sulpiride, per se, did not influence spontaneous muscular activity in SHAM preparations ($-11 \pm 2\%$; $P > 0.05$, Fig. 4A), but significantly decreased the ileal contractile amplitude in DSS segments ($-27 \pm 3\%$, $P < 0.05$; Fig. 4B). In intestinal preparations obtained from SHAM, but not DSS, mice, dopamine (30 μM) inhibited the 4-Hz EFS-induced cholinergic contraction amplitude ($-36 \pm 2\%$, $P < 0.05$) (Fig. 4C and D). In SHAM conditions, SCH-23390, per se, significantly increased 4-Hz EFS-induced cholinergic contraction ($+23 \pm 2\%$, $P < 0.05$) and reverted the dopamine-mediated inhibitory effect (Fig. 4C). In the same experimental group, 10 μM sulpiride, did not alter, per se, electrically induced cholinergic contractions, neither antagonized dopamine-induced inhibition (Fig. 4C). In DSS mice, the blockade of D1R or D2R enhanced the 4-Hz EFS-induced cholinergic response (by $+27 \pm 3\%$ or $+ 40 \pm 5\%$, $P < 0.05$, respectively) that, for both receptors, was not sensitive to the addition of dopamine (Fig. 4D).

3.4. DSS-induced ileitis alters the architecture of the myenteric plexus

To better characterize the impact of DSS treatment on ENS morphology, we analyzed the expression and the immunoreactivity of D1R and D2R in the myenteric plexus by qRT-PCR and confocal immunofluorescence (Fig. 5). The development of ileitis determined a marked increase of D1R immunoreactivity ($+26 \pm 2\%$; $P < 0.05$; Fig. 5A and B) associated with a 7- and 2-fold increase in D1R and D2R mRNA transcripts in ileal segments, respectively (Fig. 5C and D). Immunohistopathology of ileal sections revealed stronger DAT immunoreactivity after DSS treatment (Fig. 6A). Furthermore, LMMPs of DSS mice showed a higher DAT and S100β immunofluorescence ($+17 \pm 1\%$, $P < 0.05$; $+25 \pm 2\%$, $P < 0.05$; respectively) together with a reduced total number of HuC/D$^+$ neurons ($-10 \pm 1\%$, $P < 0.05$; Fig. 6B–F), indicative of neuropathy associated to reactive gliosis [44].

4. Discussion

IBD, such as Crohn's disease (CD) and ulcerative colitis (UC), is rising globally and causes intestinal inflammation associated to abdominal pain and diarrhea [48,49]. The etiology of IBD is still unclear, however several factors are involved, ranging from commensal microbiota to environmental triggers and hereditary factors [50]. Indeed, an excessive immune response against the self-constituents of the mucosal microbiota

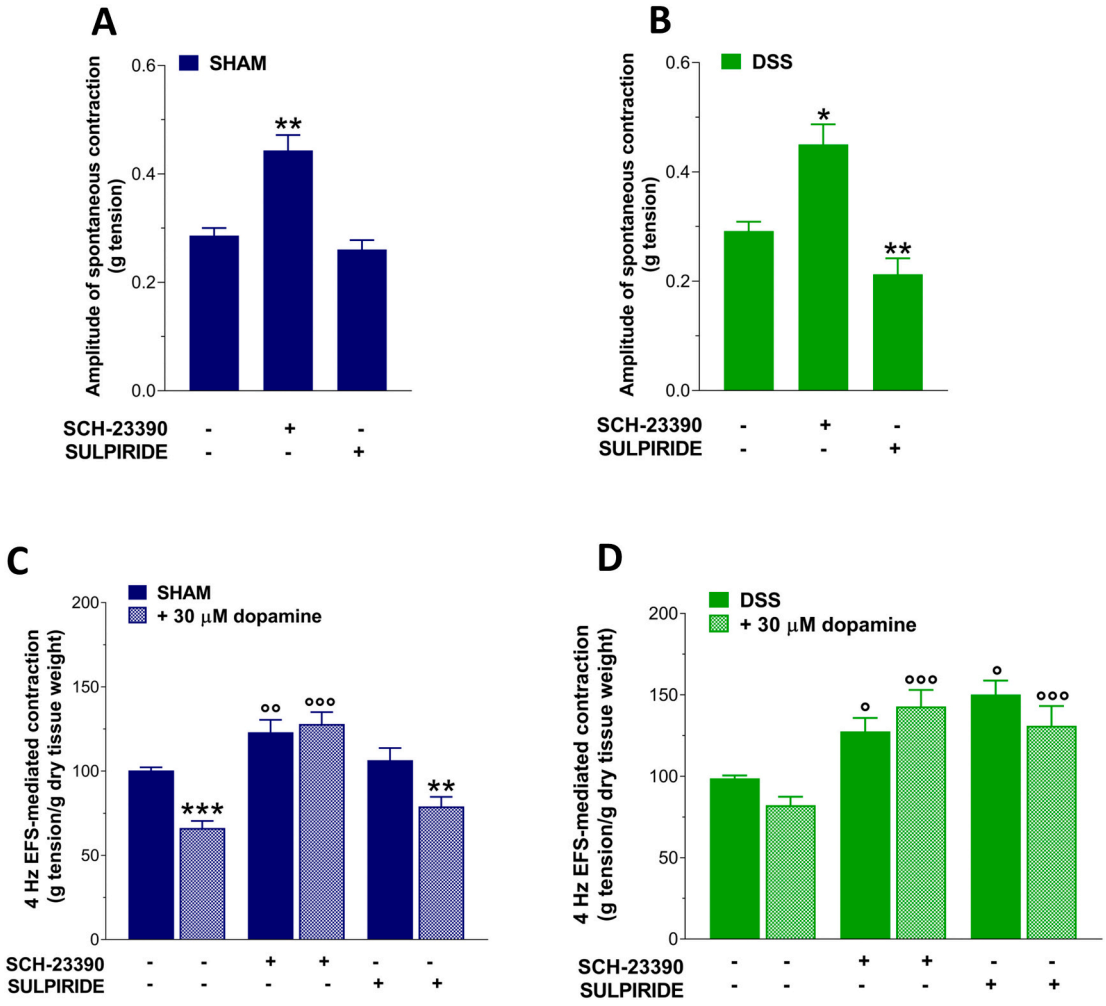

Fig. 4. DSS-induced ileitis affects D1 and D2 receptor antagonist-mediated neuromuscular response. A–B) Amplitude of spontaneous contraction without or with 10 μM SCH-23390 or 10 μM sulpiride in isolated ileal segments of SHAM (A) and DSS (B) mice. C–D) Neuromuscular excitatory response induced by 4 Hz-EFS in absence or presence of 30 μM dopamine after pretreatment with SCH-23390 or sulpiride in isolated ileal preparations of SHAM (C) and DSS (D) mice. Data are reported as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control; °$P < 0.05$, °°$P < 0.01$, °°°$P < 0.001$ vs respective control in absence of antagonists. $N = 6$ mice/group.

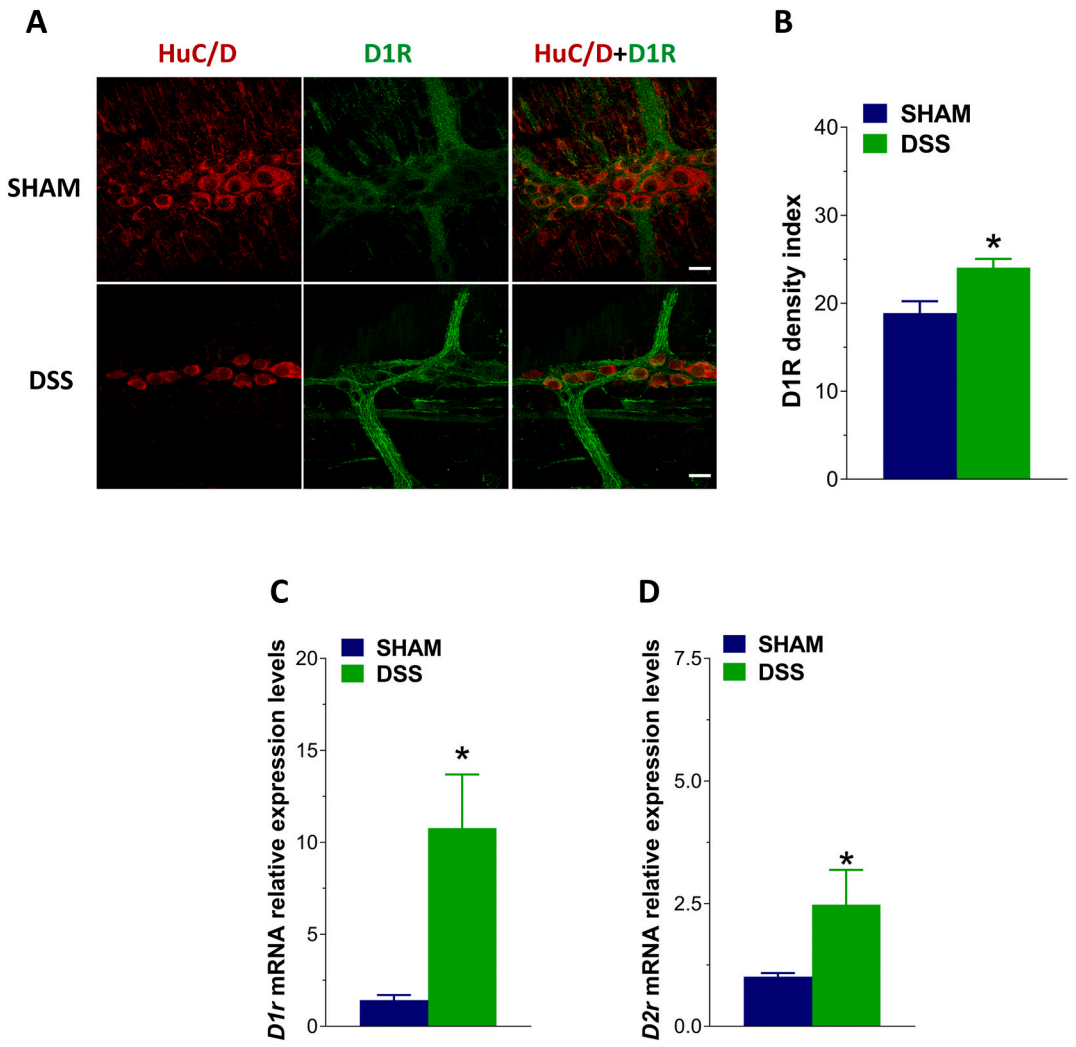

Fig. 5. DSS-induced ileitis affects dopamine receptor expression and immunoreactivity. A) Representative confocal microphotographs showing the distribution of HuC/D^{+} neurons (red), D1 receptor (D1R; green) in SHAM and DSS LMMPs (bars = 22 μm). B) Changes in D1R density index in SHAM and DSS LMMPs. qRT-PCR quantification of *D1r* (C) and *D2r* (D) mRNA levels in the small intestine of SHAM and DSS mice. Data are reported as mean ± SEM. *$P < 0.05$ vs SHAM mice. $N = 6$ mice/group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

seems to be involved in the pathogenesis of IBD [18]. Homeostasis of the gastrointestinal function relies on the highly coordinated interactions between immunity and ENS. In the enteric microenviroment, diverse immune cells and intrinsic neuronal populations mutually regulate the functional activities of the digestive system, communicating through various neurotransmitters, including dopamine, acetylcholine, serotonin and many others [51]. In this scenario, dopamine ensures gut homeostasis, acting as an inhibitory neuromodulator of gastrointestinal motility [52], and protects the mucosal barrier through regulation of submucosal blood flow and mucus secretion [53]. Data obtained from several studies suggest that the enteric dopaminergic system is one of the key players in controlling the severity of colitis [17,18,20,24,54,55]. Interestingly, a marked reduction in the ratio of dopamine/L-DOPA have been observed in the inflamed mucosa of UC and CD patients [23]. In a rat model of colitis, treatment with a D2R agonist ameliorated the severity of colitis, suggesting the potential of D2R stimulation in the control of inflammation [20]. DR1 seem also to be involved in the control of intestinal inflammation, since mice affected by DSS-induced colitis showed an attenuation of the mucosal inflammatory process after in vivo treatment with SKF38393, a D1-like agonist [56]. Although these findings are suggestive for the involvement of the dopaminergic system in IBD, the influence of dopaminergic neurotransmission during ileitis has not been clear-cut elucidated, yet. In this experimental study, we show that the induction of small intestine inflammation by DSS treatment causes: (i) an inflammatory process associated to a reduced body weight and signs of dysmotility; (ii) changes in dopaminergic inhibitory responses, primarily involving by D1-like receptor activity; (iii) an increased expression of D1R and DAT in the myenteric plexus together with substantial anomalies of the neuroglial network.

Colitis induced by DSS treatment, is a cost-effective model of IBD particularly due to its simplicity, reliability, and efficiency [57]. In our model, oral administration of DSS caused an inflammatory process in the small intestine that induced a marked reduction of body weight, associated with dysmotility and increased intestinal permeability, as suggested by reduced fecal output and higher water content in the fecal pellets, respectively, and enhancement of pro-inflammatory cytokine levels in the gut wall [27,58,59].

Exogenous dopamine peripherally affects ileal contractility by activating D1- and D2-like receptors. D2R knock-out mice displayed an increased gastrointestinal transit, suggesting that enteric neuronal D2R are involved in dopamine-mediated inhibition of intestinal motility [3]. More recently, our group and others have shown that in normal condition dopamine determines relaxation of the mouse small intestine through activation of D1Rs [2,4,60]. In our mouse model of ileitis, non-cumulative addition of exogenous dopamine caused a significant reduction of ileal spontaneous amplitude. Since D1R and D2R are the

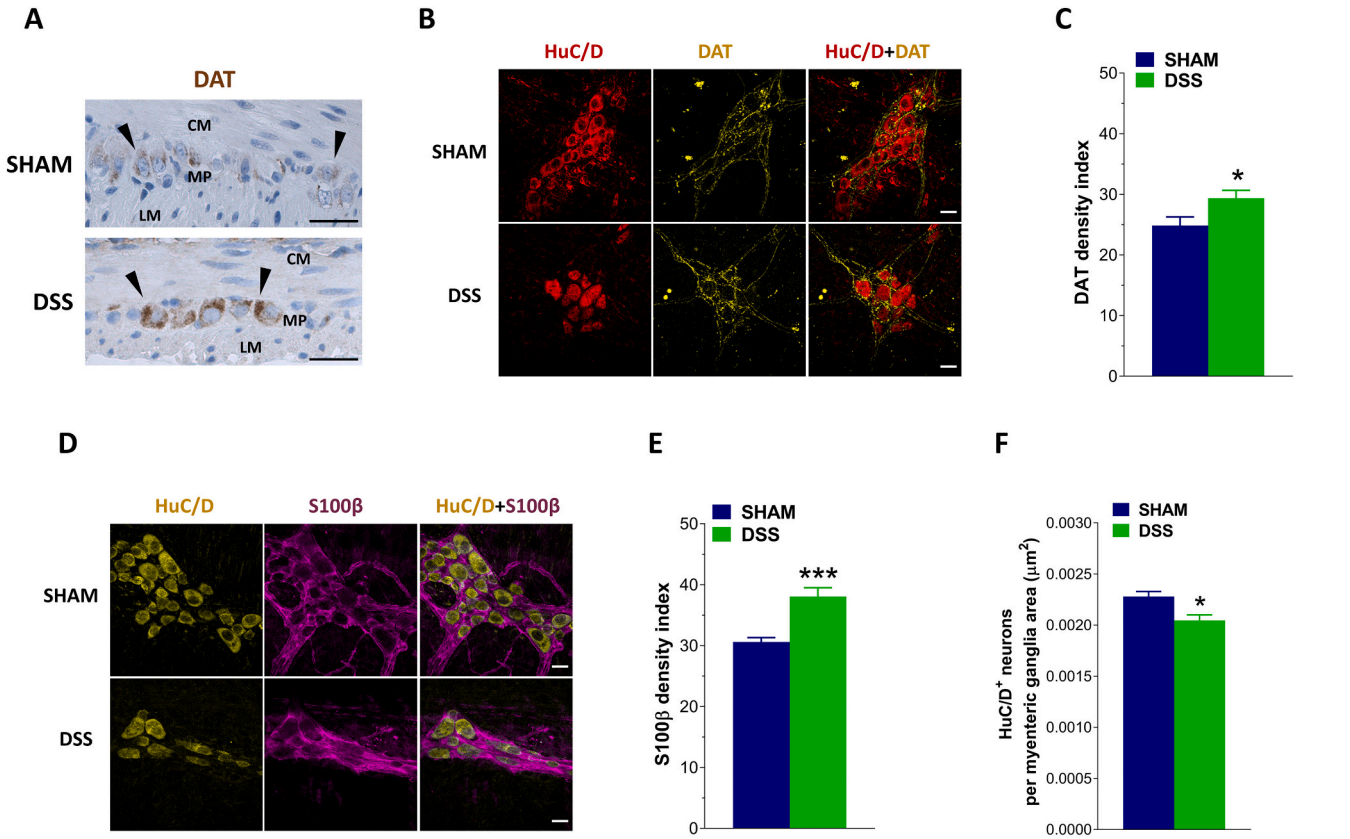

Fig. 6. DSS-induced ileitis affects dopaminergic pathways and neuroglia network. A) DAT immunostaining in ileal neuromuscular sections of SHAM and DSS mice. Scale bars = 35 μm. In myenteric ganglia DAT$^+$ neurons are marked with a black arrowhead. LM = longitudinal muscle; MP = myenteric plexus; CM = circular muscle. B) Representative confocal microphotographs showing the distribution of HuC/D$^+$ neurons (red), DAT (yellow) in SHAM and DSS LMMPs (bars = 22 μm). C) Changes in DAT density index in SHAM and DSS LMMPs. D) Representative confocal microphotographs showing the distribution of HuC/D$^+$ neurons (yellow), and S100β (magenta) in SHAM and DSS LMMPs (bars = 22 μm). E) Changes in S100β density index in SHAM and DSS LMMPs. F) Analysis of HuC/D$^+$ neurons in ileal LMMPs of SHAM and DSS mice. Data are reported as mean ± SEM. *$P < 0.05$, ***$P < 0.001$ vs SHAM mice. $N = 6$ mice/group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

major functional subtypes of DRs in gut tissue [2,4], we incubated our preparations with either SCH-23390 or sulpiride. The benzazepine derivative, SCH-23390, owing to its >500-fold D1:D2 selectivity and very low affinity for most other neuroreceptors, is a very useful pharmacological tool to characterize D1-like receptor-mediated effects [61]. Instead, sulpiride has a 200-fold greater affinity for D2 receptors over α2-adrenergic receptors, and is considered a potent D2 receptor antagonist [2].

In control mice, exogenous dopamine addition inhibited the amplitude of spontaneous phasic ileal activity as well as 4-Hz-EFS-induced cholinergic contractions [34]. The addition of SCH-23390 to ileal preparations from WT and DSS mice determined a significant and reversible increase in the amplitude of the spontaneous contractile activity, as previously observed in normal conditions [2,4]. On the other hand, sulpiride caused a significant and reversible reduction of the contraction amplitude only in DSS preparations, with no effects in SHAM segments.

In SHAM ileal preparations, the addition of dopamine resulted in about 35% decrease of 4-Hz-EFS-mediated contraction, which was totally reverted following D1R blockade but not by D2R inhibition, advocating that the mouse ileum neuromuscular function is under control of a physiologically dopaminergic inhibitory tone, as previously observed [2,12]. In DSS mice, preincubation with submaximal concentrations of dopamine did not affect 4-Hz-EFS-induced contractions, confirming the decreased pharmacological efficacy during ileitis. Surprisingly, ileitis affected D2R activity, since, at variance with the effect observed in sham preparations, addition of sulpiride determined a higher evoked mechanical activity, insensitive to dopamine addition. In this condition we cannot exclude the involvement of D1-like receptor signaling. Examination of the propulsive gastrointestinal activity in transgenic D2R deficient mice has suggested that endogenous dopamine may act via axonal D2R, causing the inhibition of acetylcholine release from enteric neurons [3,16]. In fact, anti-dopaminergic drugs (i.e., domperidone and sulpiride), acting as blockers of enteric (neuronal and muscular) inhibitory D2R, improve the strength of neurotransmission in prokinetic pathways, promoting a faster gastrointestinal transit [11].

Considering the pathophysiological relevance of dopamine pathways in gut inflammatory processes, we further focused our attention on the mouse myenteric neuroglial network, which has a crucial role in the propagation of microinflammatory processes predisposing to neuroplastic adaptive changes. In the mouse small intestine myenteric plexus, ileitis was associated with higher D1R transcript levels and immunoreactivity, and with significant changes in D2R mRNA levels, highlighting the key role of both DRs during enteric inflammation. Dopamine is recognized to be a neuroimmune modulator that controls NLRP3 inflammasome activation through D1R signaling in macrophages [56,62]. Interestingly, the increased D2R expression in the colonic mucosa of experimental models of colitis (i.e., iodoacetamide, genetic deficiency of IL-10) was associated to enhanced vascular permeability and vascular leakage [20]. Beside neuronal cells, peripheral immune cells as well as microglia and astrocytes express functional DRs to control immune cell functions and possess all the specific proteins for dopamine synthesis, metabolism, and storage to control its tissue levels [63]. Therefore, we evaluated dopamine pathways by determining the

distribution of DAT, the transporter responsible of the neuronal reuptake of the intrasynaptic excess of the neurotransmitter [4,64]. In mice carrying defective DAT expression we recently demonstrated enhanced intestinal motor responses induced by exogenous dopamine, mainly via D1R involvement. Such functional changes were associated to a higher number of HuC/D$^+$ neurons and increased glial protein S100β staining [4]. In this study, LMMP preparations from DSS mice displayed a significant increase of DAT immunoreactivity, possibly associated with a higher dopamine reuptake. These alterations were accompanied by a reduced number of HuC/D$^+$ neurons with an increased staining of the glial protein S100β, evocative of reactive gliosis. Interestingly, downregulation of myenteric neuron number with reactive gliosis has been observed in a number of different pathophysiological condition of the gastrointestinal tract, including enteric dysbiosis [38], diet-induced obesity [65–68], neurodegeneration [69] and IBD [70].

5. Conclusion

Our study shows that DSS treatment determined a significant reduction of dopamine-mediated relaxation, involving primarily D1-like receptor, accompanied by increased DAT and D1R immunoreactivity as well as higher D1R and D2R mRNA levels in the myenteric plexus. These findings underline the undeniable and complex role of dopamine in small intestine motor disturbances, and specifically its direct involvement in inflammation-induced neuromuscular alterations. Indeed, the localization of DRs and the impact of the dopaminergic system in the gastrointestinal tract is still far behind the advancements in the central nervous system. Until now, limited attention has been paid to the neuroimmune interactions, which could further connect IBD and PD pathogenesis and clarify the possible role of dopamine in order to identify target elements in the dopaminergic machinery for the treatment, not only of IBD, but possibly of other inflammatory diseases involving brain centers, via the so-called gut–brain axis [71–73].

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CRediT authorship contribution statement

Silvia Cerantola: Conceptualization, methodology, formal analysis, writing - original draft preparation, writing - review and editing; **Sofia Faggin:** Conceptualization, methodology, formal analysis, writing - original draft preparation, writing - review and editing; **Valentina Caputi:** Conceptualization, methodology, formal analysis, writing - original draft preparation, writing - review and editing; **Annalisa Bosi:** Methodology, formal analysis; **Davide Banfi:** Methodology, formal analysis; **Anna Rambaldo:** Methodology, formal analysis; **Andrea Porzionato:** Conceptualization, Methodology, writing - original draft preparation, writing - review and editing; **Rosa Di Liddo:** Conceptualization, Methodology, writing - original draft preparation, writing - review and editing; **Raffaele De Caro:** Methodology, formal analysis, writing - review and editing; **Edoardo V. Savarino:** Conceptualization, writing - original draft preparation, writing - review and editing, supervision, funding acquisition; **Cristina Giaroni:** Conceptualization, methodology, writing - original draft preparation, writing - review and editing, supervision, project administration, funding acquisition; **Maria Cecilia Giron:** Conceptualization, methodology, writing - original draft preparation, writing - review and editing, supervision, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

Edoardo V. Savarino has received lecture or consultancy fees from Abbvie, Alfasigma, Amgen, Bristol-Myers Squibb, EG Stada Group, Fresenius Kabi, Grifols, Janssen, Innovamedica, Malesci, Medtronic, Merck & Co, Novartis, Reckitt Benckiser, Sandoz, SILA, Sofar, Takeda, Unifarco.

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